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# Human Papillomavirus Type 32 Does Not Display *in Vitro* Transforming Properties

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Human papillomavirus type 32 (HPV32) is one of the etiological agents of a benign oral condition, focal epithelial hyperplasia. However, the previously characterized properties of its E7 oncoprotein suggest a possible malignant nature for this virus. In this study we characterized the properties of HPV32 E6 and E7. Our data show that HPV32 E7, despite its high affinity for pRb, does not promote degradation of the cellular protein. In addition, HPV32 E6 does not prevent p53-mediated apoptosis and/or cell cycle arrest. Moreover, coexpression of HPV32 E6 and E7 in primary human fibroblasts or keratinocytes does not alter their proliferative state. Together, these data provide evidence of the benign nature of HPV32. © 2002 Elsevier Science (USA)

*Key Words:* focal epithelial hyperplasia; HPV32; E6 and E7; cell cycle and apoptosis.

## INTRODUCTION

The human papillomavirus (HPV) family comprises over 100 different genotypes, which can be divided into two subgroups, cutaneous and mucosal, according to their tissue tropism (de Villiers, 1997; zur Hausen, 2000). Both HPV groups are further separated into benign and malignant types, based on the nature of the lesion induced. The mucosal HPV16 is a high-risk, very common type that has been detected in genital tract cancers as well as in a subgroup of head and neck cancers (zur Hausen, 2000). Two early proteins, E6 and E7, play a major role in the transformation of host cells (Tommasino, 2001). Key events in HPV-mediated transformation are the inactivation of the tumor suppressors p53 and pRb by E6 and E7, respectively, leading to deregulation of fundamental cellular events, such as apoptosis and proliferation (Tommasino, 2001).

Several studies have demonstrated that the efficiency of E6 and E7 from different HPV types in inducing *in vitro* cellular transformation strongly correlates with the *in vivo* viral-induced events. For instance, the E6 and E7 of the low-risk HPV types, which are mainly associated with benign lesions, interact only weakly with the two tumor suppressors and are not able to efficiently immortalize primary human keratinocytes (Halbert *et al.,* 1992; Munger *et al.,* 1989). In contrast, the high-risk HPV16 E6 and E7 are highly active in both events (Tommasino, 2001).

HPV13 and HPV32 are the etiological agents of a benign oral condition termed focal epithelial hyperplasia (FEH) (reviewed in Syrjanen and Syrjanen, 2000). This type of lesion was initially found to be most prevalent in the infant and juvenile population of restricted geographical areas (reviewed in Syrjanen and Syrjanen, 2000). However, in the last 10 years this disease has been reported more often in adults, particularly as an opportunistic infection in HIV patients (Marvan and Firth, 1998). In a previous study we have observed that HPV32 and HPV16 E7 proteins share several *in vitro* properties. Both proteins bind pRb with approximately the same efficiency and transform immortalized rodent fibroblasts, which acquire the ability to grow in the absence of mitogenic signals and in an anchorage-independent manner (Caldeira *et al.,* 2000). These molecular features of HPV32 and the fact that FEH is increasing in adults (e.g., HIV patients), who are frequently exposed to oral carcinogens, raise the question of whether HPV32-induced lesions may be a precondition for oral carcinogenesis. To address this question we have characterized in detail the biological properties of HPV32 E6 and E7 in immortalized and primary cells. Our data indicate that HPV32 does not represent a risk factor for tumor development.

## RESULTS

### HPV32 E7 and pRb pathway inactivation

To determine the absolute binding affinity of HPV32 E7 protein to pRb, we performed a plate-binding assay as previously described (Dong *et al.,* 2001). Figure 1A and Table 1 show that HPV32 E7 has a very high binding affinity for pRb with a dissociation constant ($K_D$) smaller than the HPV16 E7 $K_D$. These findings are in agreement

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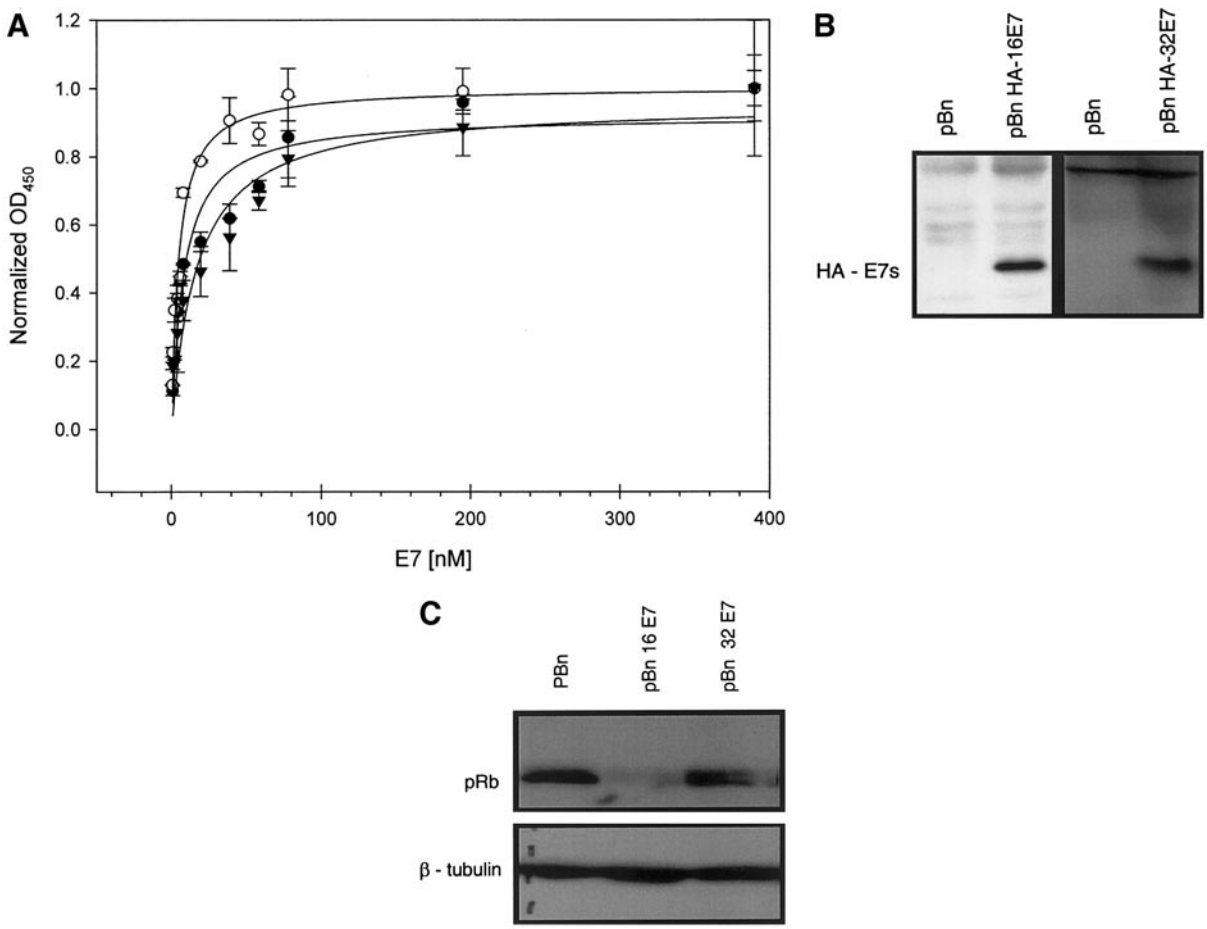


FIG. 1. HPV32 E7 does not efficiently inactivate the pRb pathway. (A) Ability of HPV32 E7 to associate with pRb in the plate binding assay. After binding of pRb to the plate, purified T7-fused recombinant HPV16 E7 (closed circles), pRb binding deficient HPV16 E7 C24G mutant (triangles), and HPV32 E7 (open circles) were added to the plate in increasing concentrations (0.7 nM–0.4 $\mu$M). Bound T7-E7 was quantified as described (Dong *et al.,* 2001). The line shows the fit of the experimental data with a single rectangular hyperbola (Sigma plot) from which the E7 concentration to obtain 50% pRb binding ($BC_{50\%}$) and the $K_D$ were determined. The OD represents the normalized average of duplicate determinations. Bars represent SD. (B) Expression of HA tag E7 proteins in NIH3T3 cells. One hundred micrograms of protein extract was fractionated on a 15% polyacrylamide–SDS gel, transferred to PVDF membrane, and incubated with an anti-HA tag monoclonal antibody (MMS-101R, Babco, 1/1000). (C) HPV32 E7 does not induce pRb degradation. One hundred micrograms of protein extracts of cells expressing the different viral proteins as indicated was applied to an 8% polyacrylamide–SDS gel, transferred onto a PVDF membrane, and incubated with an anti-pRb (14001A; Pharmigen) or $\beta$-tubulin (TUB2.1, Sigma) antibody. The $\beta$-tubulin signal was used as a loading control.

TABLE 1

Sequence of the pRb Binding Domain and Affinity of Different E7 Proteins

| HPV type | pRb binding domain | $BC_{50}$ (nM) | $K_D$ (M) |
|---|---|---|---|
| 16 | TTD**LYCYE**QLN | 4.5 | $4.5 \times 10^{-9}$ |
| 16 C24G | TTD**LYGYE**QLN | 8.9 | $8.9 \times 10^{-8}$ |
| 32 | PVD**LYCYE**QFD | 2.4 | $2.4 \times 10^{-9}$ |

*Note.* $BC_{50}$ corresponds to binding concentration at 50% saturation and the $K_D$ was calculated as described (Dong *et al.,* 2001). Amino acids in boldface indicate the core of the pRb binding domain. The HPV16 E7 C24G harbors a mutation in the pRb binding site and has a reduced affinity for the cellular protein.

with our previous data, which showed that HPV32 E7 is more efficient than HPV16 E7 in binding pRb in the yeast two-hybrid assay (Caldeira *et al.,* 2000).

HPV16 E7 association with pRb leads to the rapid degradation of the cellular protein via the proteasome pathway (Tommasino, 2001). Therefore, we determined whether HPV32 E7 is able to degrade pRb. We expressed HPV16 and HPV32 E7 proteins in NIH3T3 cells using retroviral vectors (pBabe). In order to detect the viral proteins, the hemagglutinin tag (HA tag) was fused in frame at the N-terminal of both E7s. Figure 1B shows that both proteins were expressed in rodent cells. As expected, no pRb was detected in cells expressing HPV16 E7 (Fig. 1C). In contrast, HPV32 E7 cells have intracellular levels of pRb similar to the mock cells (Fig. 1C). Thus,

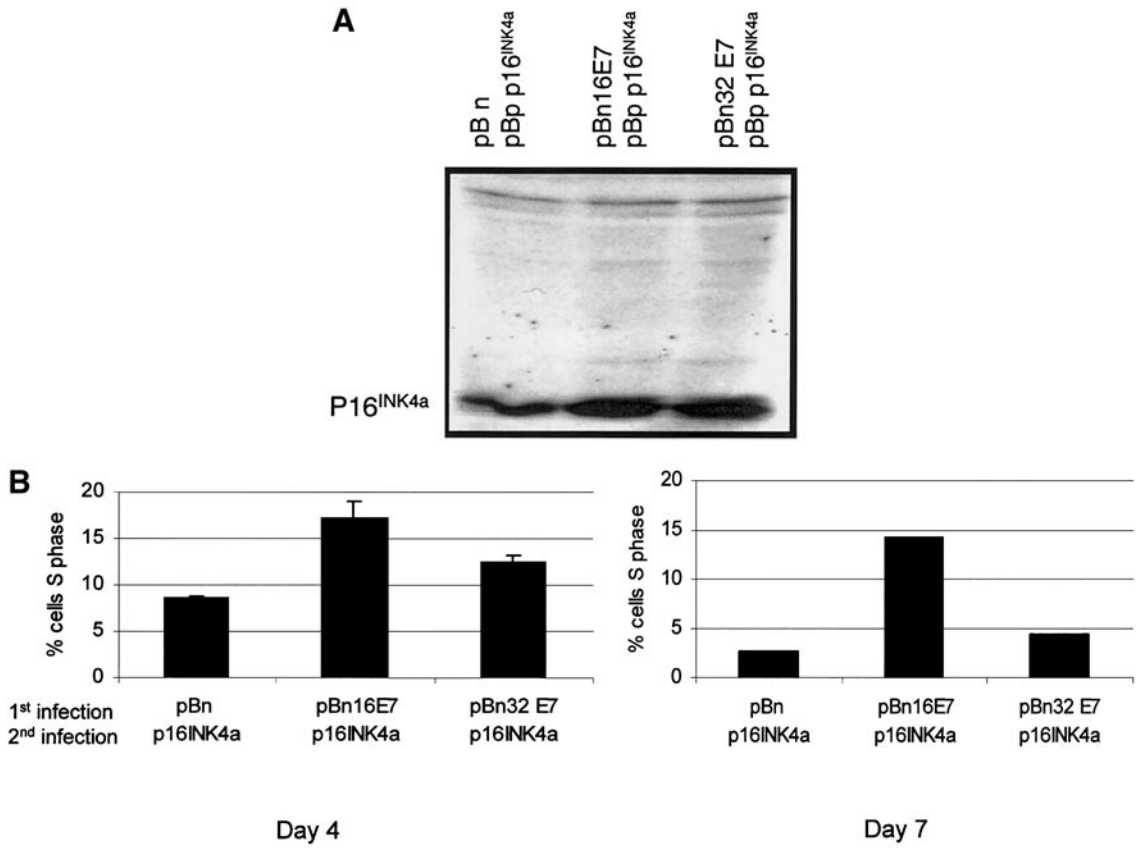


FIG. 2. HPV 32 E7 does not efficiently neutralize the inhibitory activity of p16$^{INK4a}$. (A) Expression of p16$^{INK4a}$ in NIH3T3 cells. One hundred micrograms of protein extracts of cells infected with different recombinant retroviruses as indicated was applied to a 15% polyacrylamide–SDS gel, transferred onto PVDF membrane, and incubated with antihuman p16$^{INK4a}$ antibody (kindly provided by Gordon Peters, Imperial Cancer Research Fund, London, United Kingdom). (B) Percentage of cells coexpressing E7 proteins and p16$^{INK4a}$ in S phase. Cells were double infected with different recombinant retroviruses as indicated, harvested 4 (left) or 7 (right) days postinfection with p16$^{INK4a}$ recombinant retrovirus, and stained with propidium iodide. The cell cycle profile was analyzed by flow cytometry. The data represent the mean of three independent experiments. The bars represent the standard deviations of the values obtained in independent experiments.

HPV32 E7, despite its high affinity for pRb, is not able to promote its degradation. Similar results were obtained with the E7 native proteins, thus excluding any possibility of HA tag interference (data not shown).

## HPV32 E7, G1/S progression, and CDK inhibitor p16$^{INK4a}$

We have recently reported that degradation of the retinoblastoma protein by HPV16 E7 contributes to an efficient abrogation of the cell cycle arrest imposed by overexpression of p16$^{INK4a}$ (Giarrè *et al.,* 2001). Since HPV32 E7 does not degrade pRb, we asked whether its strong binding to pRb might interfere with the pRb pathway through other means. Therefore, we determined its ability to promote G1/S progression in the presence of high levels of p16$^{INK4a}$. For this purpose, NIH3T3 cells were double infected with recombinant retroviruses expressing the HPV16 or HPV32 E7 gene and p16$^{INK4a}$ as previously described (Giarrè *et al.,* 2001). Western blot analysis revealed that p16$^{INK4a}$ was efficiently expressed (Fig. 2A). In order to evaluate the ability of HPV32 E7 to suppress the growth-inhibitory function of p16$^{INK4a}$ we then determined the percentage of S phase cells in the different cultures by flow cytometry. As shown in Fig. 2B, HPV32 E7/p16$^{INK4a}$ cells showed a low level of proliferation, similarly to the cells expressing p16$^{INK4a}$ alone. This is particularly evident after 7 days postinfection (Fig. 2B, right). In contrast, HPV16 E7 was able to counteract the inhibitory function of p16$^{INK4a}$. Thus, HPV32 E7 does not efficiently overcome the cell cycle arrest imposed by high levels of p16$^{INK4a}$.

## HPV32 E6- and p53-mediated pathways

HPV16 E6 is able to bind and degrade p53, abrogating the p53-mediated apoptotic and quiescent events (Mantovani and Banks, 2001). To determine whether HPV32 E6 has this property, we performed a GST pulldown assay using GST/E6 bacterial recombinant protein and *in vitro* translated human p53. Figure 3A shows that in contrast to HPV16 E6, HPV32 E6 is unable to associate

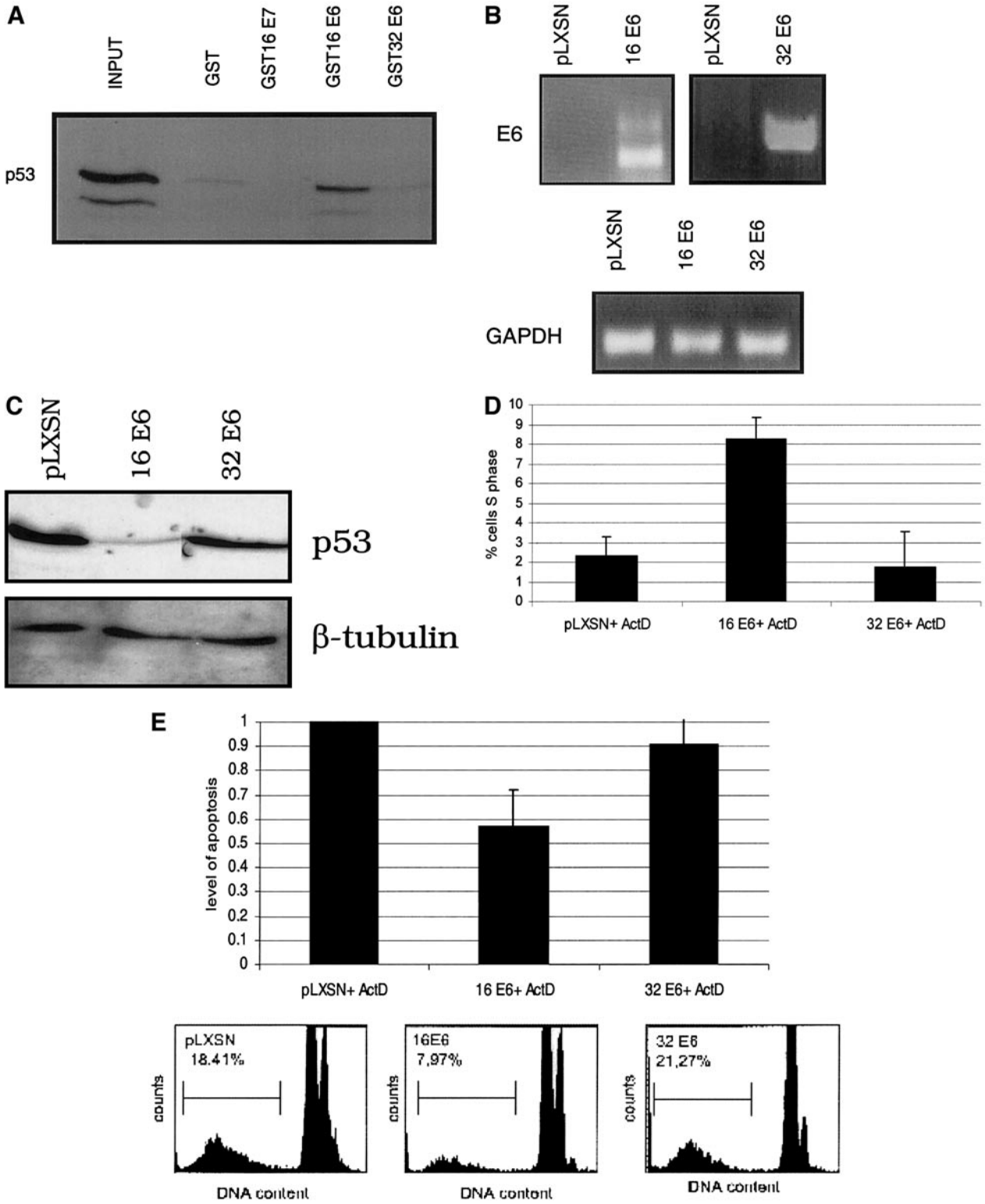


FIG. 3. HPV32 E6 does not interfere with p53 functions. (A) HPV 32E6 does not bind p53 in a GST pulldown assay. Approximately 1 $\mu$g of the different E6 proteins N terminally fused to GST was immobilized on glutathione–Sepharose beads and incubated with *in vitro* translated and radiolabeled p53. After 3 h, the beads were extensively washed, resuspended in SDS–sample buffer, and applied to a 10% polyacrylamide–SDS gel; 1/20 of the total *in vitro* translated p53 used in the GST pulldown assay was also loaded on the gel (input). GST and GST16E7 were used as negative controls. (B) The viral oncogenes are efficiently transcribed in primary oral fibroblasts. The cDNA was generated as described under Materials and Methods. Primers used for PCR amplification flanked the ORFs (see Material and Methods). cDNA obtained from cells infected with empty pLXSN retrovirus was probed with all the different primers and used as a control for the specificity of the amplified product. As an internal control, the GAPDH cDNA was also amplified. The products were separated on 1% agarose gels and visualized by staining with ethidium bromide. (C) HPV32 E6 does not

with p53. To further investigate the ability of HPV32 E6 to interfere with p53 functions, we expressed the viral protein in primary human fibroblasts (POFs) and examined its impact upon the p53 pathways. Expression of the viral genes was confirmed by RT–PCR (Fig. 3B). Several studies have demonstrated that HPV16-infected cells, in addition to the full-length E6/E7 polycistronic mRNA, contain different transcripts generated by alternative splicing (termed E6*/E7) comprising truncated forms of the E6 gene fused to the full-length E7 gene (reviewed in Tommasino, 2001). Thus, the different bands detected in HPV16 E6 expressing cells (Fig. 3B) are most likely due to this event. Western blot analysis revealed that cells expressing HPV32 E6 contained similar p53 levels to mock cells, showing that this E6 protein does not target p53 for degradation (Fig. 3C). In contrast, very low levels of the tumor suppressor were detected in HPV16 E6 cells (Fig. 3C). To determine whether p53 retains its biological functions in the presence of HPV32 E6, we analyzed the p53-mediated events in response to cellular stresses in POFs expressing the viral gene. Actinomycin D is a DNA intercalator and induces p53 accumulation, which in turn triggers cell cycle arrest and/or apoptosis (Ljungman *et al.,* 1999). Actinomycin D treatment led to cell cycle arrest in mock cells as well as in cells expressing HPV32 E6, while HPV16 E6 POFs continued to proliferate (Fig. 3D). The cell cycle arrest correlates with high levels of p53 and the cell cycle inhibitor $p21^{cip1/waf1}$, a p53 transcriptional target (data not shown). Similarly, HPV32 E6 was not able to prevent apoptosis induced by actinomycin D (Fig. 3E). Together, these data show that HPV32 E6 is not able to alter the functions of p53.

### Activities of HPV32 E6 and E7 in primary human cells

Our data show that HPV32 E6 or E7 alone does not possess *in vitro* transforming activity. However, studies on HPV16 have shown that the transforming activity of E7 is greatly increased in primary cells by the presence of E6 (Halbert *et al.,* 1991; Watanabe *et al.,* 1989), reflecting the natural situation in a viral infection. Therefore, we next analyzed the events induced by coexpression of the HPV32 E6 and E7 genes in primary human fibroblasts. To determine whether the viral genes are expressed in these cells, we performed RT–PCR analysis (Fig. 4A). Similarly to the data presented in Fig. 3B, we detected more than one transcript in cells expressing the HPV16 genes (Fig. 4A), while a single band was observed in the

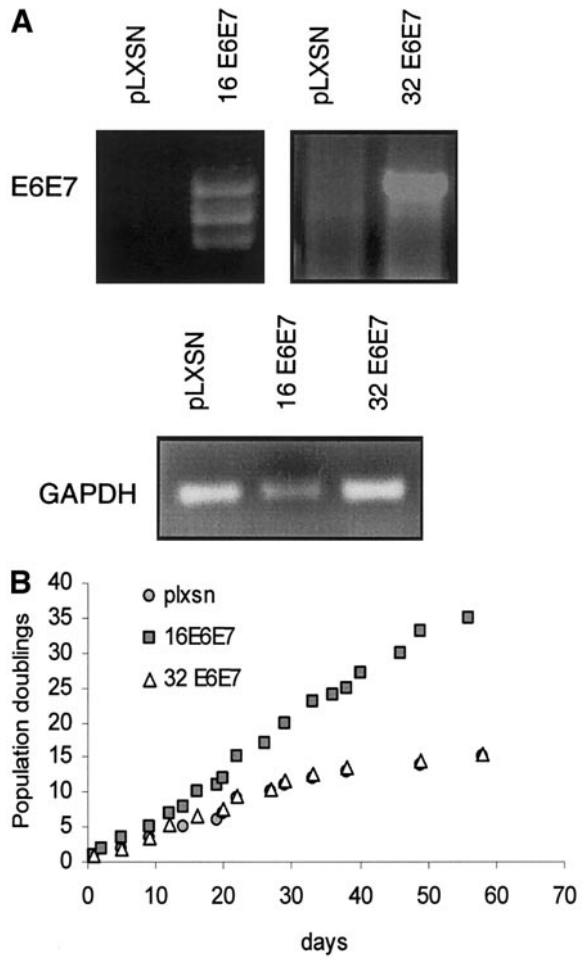


FIG. 4. Coexpression of HPV32 E6 and E7 in human fibroblasts does not alter their proliferative state. (A) Expression of the viral genes in POFs. The expression of the viral genes was determined using RT–PCR as described above. (B) Growth curves of POFs expressing the different viral proteins. The growth of the different cell lines indicated in the figure was monitored for more than 50 days. Cells were split at ~80% confluency. The doubling populations were calculated considering the split ratio. Day 0 represents the first day after selection in neomycin.

case of HPV32. The fibroblasts expressing both HPV32 genes did not significantly differ in their growth from the mock cells, while HPV16 E6/E7 cells were able to proliferate faster (Fig. 4B). Moreover, we observed that after approximately 70 days in culture, control (pLXSN) as well as HPV32 E6/E7 fibroblasts presented the typical feature of senescent cells, e.g., positive $\beta$-galactosidase staining at pH 6.0 (Dimri *et al.,* 1995) (data not shown). In contrast, fibroblasts containing HPV16 E6 and E7 contin-

degrade p53 in POFs. One hundred micrograms of protein extracts of POFs expressing the different oncoproteins as indicated were separated on a 10% polyacrylamide–SDS gel, transferred onto a PVDF membrane, and incubated with anti-p53 (Dako) or $\beta$-tubulin (TUB2.1, Sigma) antibody. (D and E) Induction of cell cycle arrest and apoptosis by DNA damaging agents in HPV32 E6 expressing cells. POFs were treated for 24 h with 2.5 $\mu$g/ml of actinomycin D and then harvested, fixed in 70% ethanol, and stained with propidium iodide. The percentage of the population in S phase (D) or apoptosis (E) was determined by flow cytometry. The level of apoptosis is represented as the average of five independent experiments. The percentage of apoptotic cells in the empty vector cell line (pLXSN) was taken as a standard (1) and all the other values were normalized accordingly. The bottom panel shows a representative experiment. The apoptotic cells are visible under the bar.

TABLE 2

Infection of Primary Human Oral Keratinocytes with Retroviruses Expressing HPV16 E6/E7 and HPV32 E6/E7

| Cell line | I | II |
|---|---|---|
| PLXSN | (<1 week)[a] | (<1 week)[a] |
| HPV16 E6/E7 | Cultured for 8 months | Cultured for 8 months |
| HPV32 E6/E7 | (<1 week)[a] | (<1 week)[a] |

*Note.* I and II represent two independent infections. HPV16 E6/E7 keratinocytes were frozen at passage 22, 8 months after infection.

[a] The death of the culture.

ued to proliferate without entering replicative senescence. Next, we examined the effects of HPV32 E6 and E7 expression in the natural host cells, oral primary human keratinocytes. In two independent experiments we observed that HPV16 E6 and E7 efficiently extended the life span of the primary keratinocytes, while HPV32 E6/E7 were not able to do so (Table 2). Thus, our data demonstrate that coexpression of HPV32 E6 and E7 does not augment the activity of single proteins in altering cellular proliferation.

## DISCUSSION

In this study, we performed a detailed analysis of the biological properties of HPV32 E6 and E7 in order to evaluate the oncogenicity of this virus. We demonstrate that HPV32 E7, despite its high affinity of binding to pRb, is not able to induce its degradation. This feature is not exclusive to HPV32 E7. For example, E7 from the cutaneous type HPV1, which is normally associated with benign skin lesions, binds pRb with the same efficiency as HPV16 E7 (Ciccolini *et al.,* 1994) but does not promote pRb degradation (Giarrè *et al.,* 2001). We have previously shown that pRb degradation by HPV16 E7 protein is essential to overcome the G1 cell cycle arrest imposed by the cyclin-dependent kinase inhibitor $p16^{INK4a}$ (Giarrè *et al.,* 2001). Consistent with this observation, HPV1 E7 has a very low efficiency in neutralizing the $p16^{INK4a}$ inhibitory function (Giarrè *et al.,* 2001). Here, we show that HPV32 E7 behaves similarly to HPV1 E7, providing further support for the link between E7-mediated pRb degradation and deregulation of the G1/S transition. Like HPV32 E7, HPV1 E7 displays transforming properties in immortalized rodent fibroblasts, which do not express $p16^{INK4a}$ (Schmitt *et al.,* 1994). However, both E7s failed to transform primary cells (Ciccolini *et al.,* 1994; Schmitt *et al.,* 1994). Together, the results obtained with HPV1 E7 and HPV32 E7 indicate that pRb degradation represents a key step in the inactivation of $p16^{INK4a}$ and suggest that these activities are exclusive to oncogenic HPV types.

Unlike HPV16 E6, we have shown that HPV32 E6 does not bind or degrade p53. Thus, the p53 tumor suppressor activities are not altered in HPV32 E6 expressing cells. Indeed, in these cells, when p53 is induced by cellular stresses, it is still able to prompt apoptosis and arrest the cell cycle. Histochemical analysis reveals that the most prominent feature of FEH lesions is the presence of atypical cells, termed FEH cells, easily identifiable by their apoptotic features (Syrjanen and Syrjanen, 2000). Taking these observations together, it is likely that *in vivo,* during the course of the disease, the cellular defense mechanism leads to eradication of HPV32-infected cells by apoptosis without any resistance from HPV32 E6.

Our experiments in primary human cells provide an additional explanation for the non-oncogenicity of the virus. Coexpression of HPV32 E6 and E7 in primary fibroblasts or keratinocytes does not alter the proliferative state of the host cells. Increased expression and/or activity of p53, $p21^{CIP1/WAF1}$, and $p16^{INK4a}$ are detected in senescent cells (Bringold and Serrano, 2000), indicating the importance of the integrity of the p53 and pRb pathways in this event. Since HPV32 E6 and E7 proteins are not able to neutralize the tumor suppressors pRb and p53, these are still able to exert their activities, driving the cells into senescence, limiting their proliferative capacity, and preventing immortalization and transformation.

In summary, our data show that HPV32 is a benign type and does not represent a risk in HPV-mediated carcinogenesis.

## MATERIALS AND METHODS

### Plate binding assay

The assay was performed as described (Dong *et al.,* 2001). The E7 genes were cloned in frame and downstream of the T7 tag sequence of the pET-21a$^+$ expression vector (Novagen/Calbiochem, Darmstadt, Germany). For production of the GST-pRb fusion protein, part of the pRb gene comprising the E7-interacting region (pocket domain, amino acids 373–929) was cloned into the pGEX2T vector (Amersham Pharmacia, Freiburg, Germany).

### Retroviral expression vectors

The retroviral vectors pBabe-puro and pBabe-neo have previously been described (Morgenstern and Land, 1990), while pLXSN was obtained from Clontech (Palo Alto, California). The single open reading frames of HPV16 and HPV32 E6 were cloned into pBabe-neo with or without the HA tag sequence at the 5′ end. HPV16 E6E7 and HPV32 E6E7 ORFs were cloned in pLXSN. The pBabe-puro $p16^{INK4a}$ construct was kindly provided by Bruno Amati (DNAX Research Institute, Palo Alto, California).

### Cell culture

NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and 4.5 g/liter of glucose, sup-

plemented with 10% calf serum (NIH 3T3). Bosc23, Phoenix, and POFs were grown in DMEM supplemented with 10% fetal calf serum. Primary human oral keratinocytes were grown in serum-free KGM medium supplemented with epidermal growth factor (0.01 $\mu$g/ml), insulin (5 $\mu$g/ml), hydrocortisone (0.4 $\mu$g/ml), bovine pituitary extract (3 mg/100 ml), and gentamycin (all from Clonetics) on plasticware previously coated with 1 ml/10 $cm^2$ of coating solution [fibronectin (10 $\mu$g/ml), Sigma; BSA (1 mg/ml), Roche; and collagen type I (30 $\mu$g/ml), IBFB, Leipzig, Germany].

### Retroviral infections

High-titer retroviral supernatants (>5 × $10^6$ IU/ml) were generated by transient transfection of Bosc23 cells (ecotropic viruses) or Phoenix (amphotropic viruses) and used to infect the cells as described previously (Pear *et al.,* 1993). After infection, NIH 3T3 cells were selected in 1 mg of G418/ml (7 to 8 days) or 2.0 $\mu$g of puromycin/ml (48 h). POFs were selected in 0.5 mg of G418 (6–7 days), while primary oral keratinocytes were selected in 0.05 mg of G418/ml (2–3 days). Coexpression of the viral genes and the cell cycle inhibitor $p16^{INK4a}$ was achieved by consecutive retroviral infection as previously described (Giarrè *et al.,* 2001).

### Determination of cellular parameters

Fluorescence-activated cell sorter (FACS) analysis was performed as previously described (Giarrè *et al.,* 2001). For the detection of apoptosis (hypodiploid DNA content), floating cells were also collected and pooled together with the trypsinized cells. For determination of apoptotic profile, the cells were left overnight at 4°C in 70% ethanol, stained with propidium iodide, and analyzed by FACS.

### Immunoblot analysis and antibodies

Total cellular extracts were prepared in lysis buffer as described (Giarrè *et al.,* 2001). Cell extracts were fractionated by electrophoresis on a SDS–polyacrylamide gel. Immunoblot analysis was performed using the following antibodies: anti-HA tag (MMS-101R, Babco, Richmond, California); anti-pRb (14001A; Pharmingen, San Diego, California); anti-$\beta$-tubulin (clone TUB2.1, Sigma, Deisenhofen, Germany); anti-$p16^{INK4a}$ (kindly provided by Gordon Peters, Imperial Cancer Research Fund, London, United Kingdom).

### RT–PCR analysis

Total RNA was extracted from mammalian cells using the RNeasy kit (Qiagen GmbH, Germany) and adding a DNase I treatment to prevent cellular DNA contamination in the PCR reaction. cDNA was synthesized using the First Strand cDNA Synthesis Kit (MBI Fermentas, Heidelberg, Germany) using a random hexamer primer and M-MuLV reverse transcriptase. The following primers were used to amplify the different viral cDNA by PCR: HPV16 E6, 5′-ATGTTTCAGGACCCACAG-3′ (forward primer) and 5′-TTACAGCTGGGTTTCTCT-3′ (reverse primer); HPV32 E6, 5′-ATGGCAAGTACTTCTGCC-3′ (forward primer) and 5′-TTCTCTGCACTGGGTAC-3′ (reverse primer); HPV16 E6/E7, 5′-ATGTTTCAGGACCCACAG-3′ (forward primer) and 5′-TTATGGTTTCTGAGAACA-3′ (reverse primer); HPV32 E6/E7, 5′-ATGGCAAGTACTTCTGCC-3′ (forward primer) and 5′-TCACTCCACGCAGGCACA-3′ (reverse primer).

### GST pulldown assay

Equal amounts of immobilized GST/recombinant proteins were incubated with *in vitro* translated $^{35}$S-labeled p53. After extensive washing steps, the amount of p53 bound to GST-HPV16 E6 was determined by electrophoresis and autoradiography.

## ACKNOWLEDGMENTS

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